MOLECULAR AND CELLULAR BIOLOGY, Aug. 1995, p. 4453–4465 Vol. 15, No. 8
0270-7306/95/$04.00+0
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The Structure and Function of $p55^{PIK}$ Reveal a New Regulatory Subunit for Phosphatidylinositol 3-Kinase

SEBASTIAN PONS, TOMOICHIRO ASANO, ERIN GLASHEEN, MONTSERRAT MIRALPEIX,† YITAO ZHANG, TRACEY L. FISHER, MARTIN G. MYERS, JR., XIAO JIAN SUN, AND MORRIS F. WHITE*

Research Division, Joslin Diabetes Center, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

Received 15 March 1995/Returned for modification 28 April 1995/Accepted 18 May 1995

Phosphatidylinositol 3-kinase (PI-3 kinase) is implicated in the regulation of diverse cellular processes, including insulin-stimulated glucose transport. PI-3 kinase is composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit. Here, we describe $p55^{PIK}$, a new regulatory subunit that was isolated by screening expression libraries with tyrosine-phosphorylated insulin receptor substrate 1 (IRS-1). $p55^{PIK}$ is composed of a unique 30-residue NH_2 terminus followed by a proline-rich motif and two Src homology 2 (SH2) domains with significant sequence identity to those in p85. $p55^{PIK}$ mRNA is expressed early during development, remains abundant in adult mouse brain and testis tissues, and is detectable in adult adipocytes and heart and kidney tissues. $p55^{PIK}$ forms a stable complex with p110, and it associates with IRS-1 during insulin stimulation. Moreover, the activated insulin receptor phosphorylates $p55^{PIK}$ in Sf9 cells, and insulin stimulates $p55^{PIK}$ phosphorylation in $CHO^{IR}/p55^{PIK}$ cells. The unique features of $p55^{PIK}$ suggest that it is important in receptor signaling.

Phosphatidylinositol 3-kinase (PI-3 kinase) is a common signaling element which plays a role in the regulation of a broad array of biological responses by activated receptors for hormones, growth factors, cytokines, and antigens (6, 13, 15, 42, 46, 48, 61). It is composed of a 110-kDa catalytic subunit (p110) associated with an 85-kDa regulatory subunit (p85) that contains one Src homology 3 (SH3) domain, homology to the breakpoint cluster region (bcr) gene, two proline-rich motifs, and two SH2 domains (11). Interestingly, p110α displays dual catalytic specificity, as it phosphorylates the D-3 position of phosphatidylinositol and its phosphorylated derivatives and serine residues in p85 and insulin receptor substrate 1 (IRS-1) (12, 32). Mammalian p110 is homologous to VPS34, a *Saccharomyces cerevisiae* PI-3 kinase which is involved in vacuolar protein sorting (49); however, the molecular role of PI-3 kinase in mammalian cells is unclear (22).

The p85 regulatory subunit has a broad potential to couple the PI-3 kinase to multiple signaling elements by employing its SH3 domain, proline-rich motifs, bcr homology region, or SH2 domains (24). Most activated receptors with tyrosine kinase activity engage the SH2 domains in p85 through phosphorylated YXXM motifs in the receptors themselves or a closely associated subunit (52). The platelet-derived growth factor receptor, one of the best-characterized systems, associates directly with the SH2 domains in p85 at a phosphorylated YMDM motif in the kinase insert region (61). Inhibition of PI-3 kinase catalytic activity with wortmannin or disruption of p85 function by site-directed mutagenesis blocks several growth factor-stimulated processes, including mitogenesis and antiapoptosis (61, 67), differentiation (27), receptor trafficking (23), chemotaxis (31, 45), membrane ruffling (29, 64), and insulin-stimulated glucose transport (7, 16, 44) and *Xenopus* oocyte maturation (8, 9). In addition, PI-3 kinase appears to be required for the stimulation of $p70^{S6k}$ by platelet-derived growth factor and insulin and probably other growth factors (7, 10, 40). On the basis of these results, PI-3 kinase plays a central role in cellular signaling.

Insulin regulates PI-3 kinase by tyrosine phosphorylation of IRS-1 and IRS-2, multipotential docking proteins which contain multiple potential tyrosine phosphorylation sites, including several YXXM motifs (41, 58). In addition to insulin and insulin-like growth factor 1 (IGF-1), some interleukins (interleukin 4 [IL-4] and IL-9), growth hormone, and alpha interferon (IFN-α) and IFN-γ stimulate tyrosine phosphorylation of IRS-1 (3, 53, 63, 68). Tyrosine-phosphorylated IRS-1 binds to the SH2 domains in various signaling proteins, including PI-3 kinase, Grb-2/SOS, nck, crk, c-fyn, and SH-PTP2 and probably others (41, 58). As a consequence of docking SH2 proteins, IRS-1 mediates multiple downstream signals during insulin stimulation, including the direct activation of PI-3 kinase and SH-PTP2 (4, 34, 54) and the stimulation of mitogen-activated protein kinase and $p70^{S6k}$ (7, 10, 40), and has at least a partial role in the regulation of mitogenesis, chemotaxis, and glucose transport (16, 31, 61). Disruption of the IRS-1 gene in mice retards intrauterine growth and causes mild insulin resistance; however, the mice are not diabetic and reproduce normally, suggesting that other pathways, including IRS-2 and Shc, may compensate for the absence of IRS-1 (2).

Since IRS-1 possesses multiple tyrosine phosphorylation sites, we reasoned that recombinant IRS-1 could be used to screen cDNA expression libraries for novel SH2 proteins. Using the cloning of receptor targets (CORT) technique, we isolated a novel 55-kDa protein containing two SH2 domains with significant identity to p85α and p85β (36). This protein, designated $p55^{PIK}$, associates with p110 and during insulin stimulation binds to IRS-1. It lacks several protein-binding domains found in the NH_2-terminal portion of p85, including

* Corresponding author. Mailing address: Research Division, Joslin Diabetes Center, 1 Joslin Pl., Boston, MA 02215. Phone: (617) 732-2578. Fax: (617) 732-2593.

† Present address: Laboratorios Alnirall, 08024 Barcelona, Spain.

the SH3 domain, the first proline-rich motif, and the bcr homology region; however, p55^{PIK} contains a unique NH_2 terminus with a potential phosphorylation site in a YXXM motif. Thus, p55^{PIK} may play a unique role in growth factor signal transduction and PI-3 kinase function.

MATERIALS AND METHODS

Preparation of the ^{32}P-IRS-1 probe. Baculovirus-produced IRS-1 was labeled by incubation with purified insulin receptor in the presence of [γ-^{32}P]ATP and Mn^{2+}. The insulin receptor was purified from Chinese hamster ovary (CHO) cells overexpressing human insulin receptor (CHO^{IR} cells) on wheat germ agglutinin agarose (Vector Laboratories) as previously described (56). Approximately 5 mg of wheat germ agglutinin-purified insulin receptor was activated by autophosphorylation during a 20-min incubation with 100 nM insulin, 50 mM [γ-^{32}P]ATP (67,000 cpm/pmol; NEN), and 5 mM $MnCl_2$ (65). IRS-1 (1 mg; 8 pmol) was added to the active kinase mixture, which was then incubated at 4°C overnight. Proteins in the reaction mixture, predominantly ^{32}P-IRS-1, were reduced at 55°C for 5 h with 100 mM dithiothreitol in 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM NaCl and 6 M guanidinium chloride and then carboxymethylated with iodoacetamide (56). The methylated and reduced ^{32}P-IRS-1 was washed several times in a Centricon-30 microconcentrator (Amicon) with 10 mM Tris-HCl (pH 7.4) containing 50 mM NaCl to remove contaminating [γ-^{32}P]ATP. ^{32}P-IRS-1 was resuspended in 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 0.05% Tween 20 to a concentration of 2×10^5 to 5×10^5 cpm/ml for use as a probe. The ^{32}P-IRS-1 in this reaction was immunoprecipitated completely with antiphosphotyrosine antibody, indicating that each labeled molecule contains phosphotyrosine.

Expression screening with recombinant ^{32}P-IRS-1. To identify IRS-1-binding proteins, an oligo(dT)-primed F442a adipocyte cDNA library prepared in Uni-Zap XR (gift from B. Spiegelman, Dana-Farber Cancer Institute) was screened with ^{32}P-IRS-1. Twenty 15-cm-diameter plates representing 500,000 plaques were overlaid with nitrocellulose filters (HATF; Millipore) that were impregnated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Bethesda Research Laboratories) and incubated for 10 h at 37°C. The filters were removed, briefly washed at room temperature with TNT buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20), and then incubated in TNT buffer containing 5% Carnation instant dry milk for 6 h. The filters were incubated overnight at 4°C with ^{32}P-IRS-1 (50 μg/ml) and then washed three times at room temperature with 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 0.01% Tween 20. The dry filters were exposed to Kodak X-AR-5 film with an intensifying screen at −70°C for 24 h. Thirty primary positive plaques were selected, and 15 remained positive during two rounds of screening with ^{32}P-IRS-1. The cDNA inserts in pBluescript were prepared by in vivo excision according to the instructions of the manufacturer (Stratagene).

RNA isolation and Northern blotting. Mouse tissues were collected from embryonic days 13 and 17 and postnatal days 4 and 30 under RNase-free conditions, placed immediately on dry ice, and stored at −70°C. Total RNA was isolated by using an Ultraspect RNA isolation kit according to the instructions of the manufacturer (Biotex). RNA was separated by agarose gel electrophoresis, and uniform loading and RNA integrity were verified by ethidium bromide staining before transfer to a Nytran membrane. Northern (RNA) blots of p55^{PIK}, p85α, and p85β were obtained by using the total RNA filters prepared in our laboratory and a commercial poly(A)$^+$ RNA blot filter from 4-week-old mice (Clontech, Palo Alto, Calif.). The filters were hybridized for 16 h at 65°C by using the Rapid-Hyb System (Amersham) in a rotating oven and washed twice at 22°C and twice at 65°C with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS). Each probe contains the full-length coding region, which did not cross-react under our hybridization conditions.

Preparation of antibodies. The anti-p55^{NT} antibody (αp55^{NT}) was prepared by immunizing rabbits with a 15-amino-acid synthetic peptide based on the unique NH_2 terminus (MPYSTELIFYIEMPD) of p55^{PIK}. The peptide was coupled to keyhole limpet hemocyanin as previously described (58). The αp55^{CFT} antibody was raised in rabbits against a 17-amino-acid peptide coupled to keyhole limpet hemocyanin containing the last 9 amino acids of p55^{PIK} and the FLAG tag sequence (HAQMPTLCR/DYKDDDDK). αp85^{PAN}, an antibody that recognizes p85α, p85β, and p55^{PIK}, was produced by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein containing the NH_2-terminal SH2 domain of p85α (4). The polyclonal αIRS-1 antibody was raised in rabbits against rat IRS-1 produced in SF9 cells (40), and αp110 was obtained from Santa Cruz Biotechnology.

Immunoprecipitation. Cells were broken in 1 ml of ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Nonidet P-40, 10% glycerol, 10 mg of aprotinin per ml, 10 mg of leupeptin per ml, 2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. For tissues, the procedure was exactly the same, except that the lysis was performed in a Teflon-glass homogenizer. Insoluble material was removed by centrifugation, and supernatants were incubated with antibody at 4°C for 1 to 2 h. The immunocomplexes were collected with protein A-Sepharose 6 MB (Pharmacia) for 1 h at 4°C. Immunoprecipitations with protein A-coupled antibodies were performed for 2 h at 4°C. Immunoprecipitates were washed three times in lysis buffer before separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

For Western blotting (immunoblotting), proteins were transferred to nitrocellulose membranes, blocked, and probed as described previously (37). Blots were incubated with Renaissance chemiluminescent reagents (NEN) and exposed to Kodak X-AR film. In some cases, blots were incubated with ^{125}I-protein A and visualized by use of autoradiography (Kodak X-AR-5 film) or a Phosphorimager (Molecular Dynamics).

Expression in Sf9 cells of p55^{PIK}, p85, p110, IRS-1, and IRβ. Recombinant baculovirus containing the cDNA for the catalytic domain (β-subunit) of the insulin receptor (IRβ) or bovine p110α was obtained from previous members of O. Rosen's laboratory (18) and M. Waterfield (62), respectively. Recombinant viruses containing the cDNAs of rat IRS-1 and murine p55^{PIK} or p85α were prepared in pBluebac or pBluebacHis, respectively (Clontech). The cDNA encoding p55^{PIK} (nucleotides 1370 to 2754) was subcloned into the pBluebacHis vector by using *Bam*HI linkers containing the FLAG tag (DYKDDDDK) at the 3′ end. Similarly, nucleotides 545 to 2748 of mouse p85α cDNA were inserted into vector pBluebacHis. The preparation of IRS-1-containing baculovirus was previously reported (39). To establish the recombinant viruses, Sf9 cells were plated on 60-mm-diameter plates and transfected with each transfer plasmid in cationic liposomes (Invitrogen, San Diego, Calif.). The virus titers were determined, and the multiplicity of infection of each virus was adjusted in order to obtain equal expression levels in Sf9 cells. Infections were routinely conducted for 48 h. Cell lysates from each infection were immunoblotted with specific antibodies to verify uniform protein expression.

Expression of p55^{PIK} in CHO cells. The coding region of p55^{PIK} (nucleotides 1370 to 2754) including the FLAG tag was subcloned from pBluebac and inserted into the CAGG expression vector under the control of a β-actin promoter (43). Parental CHO cells and CHO^{IR} cells were routinely grown in Ham's F12 medium containing 10% fetal bovine serum (56). Subconfluent monolayers were cotransfected with a calcium phosphate precipitate containing 20 μg of CAGG-p55^{FLAG} and 1 μg of pEBVHIS, which contains a hygromycin resistance gene (Invitrogen), as previously described (56). Transfected cells were selected in Ham's F12 medium containing 300 μg of hygromycin B per ml. Clones of CHO^{IR}/p55^{PIK} cells expressing p55^{PIK} (including the COOH-FLAG tag) were selected by immunoprecipitation and immunoblotting with αp55^{CFT}. The cells were labeled in 10 ml of $^{32}P_i$ (0.4 mCi/ml) (NEN) for 2 h at 37°C (57). The cells were triggered for 1 min with 100 nM insulin and then solubilized and incubated with αp55^{CFT} for 2 h at 4°C as previously described (66). ^{32}P-p55^{PIK} was immunoprecipitated from the CHO/p55^{PIK} cells without or with the human insulin receptor grown in 10-cm-diameter dishes (Nunc) as previously described (56).

Identification of the tyrosine phosphorylation sites in p55^{PIK}. A mixture of glycoproteins (2 to 5 mg) containing the insulin receptor was purified from CHO^{IR} cells on immobilized wheat germ agglutinin (Vector). The insulin receptor was activated by incubation for 15 min at 22°C in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4) containing 5 mM $MnCl_2$ and 100 μM ATP in the presence of 100 nM insulin. p55^{PIK} was immunoprecipitated from CHO^{IR}/p55^{PIK} cells with αp55^{CFT} and incubated with the activated insulin receptor in 50 mM Tris-HCl (pH 7.8) containing 0.5 M NaCl, 5 mM $MnCl_2$, 100 μM ATP, and 1 mCi of [γ-^{32}P]ATP (3,000 Ci/mmol) for 2 h. Reduced and carboxymethylated ^{32}P-labeled p55^{PIK} was separated by SDS–10% PAGE and transferred to polyvinylidene difluoride (PVDF) at 4°C at 100 V for 1 h (56). The ^{32}P-p55^{PIK} was localized by brief autoradiography, excised, incubated with 0.5% polyvinylpyrrolidone 40 in 0.1 M acetic acid at 37°C for 1 h, and then washed 10 times with water. The p55^{PIK} was digested on the PVDF with 20 μg of modified sequencing-grade trypsin (Promega) in 50 mM $(NH_4)HCO_3$ containing 5% acetonitrile for 15 h at 37°C and then incubated for 10 h with 10 μg of additional trypsin. The digest was acidified with 10 μl of 10% trifluoroacetic acid, dried overnight in vacuo, and resolved in an SDS-Tricine gel as previously described (14, 56). After electrophoresis, phosphopeptides were located by autoradiography, excised, dialyzed overnight against water, and eluted with 50% acetonitrile containing 0.05% trifluoroacetic acid. The supernatant was divided into three aliquots and dried in vacuo. One of the aliquots was stored at −20°C, and the other two were subjected to a secondary digestion with 10 μg of endoproteinase Glu-C (*Staphylococcus aureus* V8 protease) in 25 μl of $(NH_4)_2CO_3$ (pH 7.8) for 15 h at 22°C or with 10 μg of endoproteinase Asp-N (Boehringer Mannheim) in 25 μl of phosphate buffer (pH 8) for 15 h at 37°C, respectively. Peptides from the three protease digests were covalently coupled to a Sequelon AA disk (Millipore). The phosphorylation site in the phosphopeptide was deduced by manual Edman degradation as previously described (55), except that the coupling and cleavage temperature was 55°C. The radioactivity in the disk was measured just before Edman degradation was started, and then the radioactivity in the disk and in the eluate after each cycle was measured.

PI-3 kinase activity. Cells were starved in high-glucose Dulbecco modified Eagle medium for 2 h and then incubated with insulin (0 to 100 nM) or IL-4 (0 to 100 ng/ml) for 10 min. The cells were lysed in 1 ml of 20 mM Tris-HCl (pH 7.5) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4, 1% Nonidet P-40 (Sigma), and 10% glycerol; insoluble material was removed by centrifugation at 13,000 × *g* for 10 min, and the supernatant was incubated with various antibodies for 4 h at 4°C. Immune complexes were precipitated from the

supernatant with protein A-Sepharose (Pharmacia), washed, and assayed as previously described (40).

RESULTS

Expression cloning of p55^{PIK}. An F442a adipose-cell cDNA expression library was screened with ^{32}P-IRS-1. Fifteen positive clones were isolated, including one novel clone (52.1.1) which contained a unique 2.7-kbp insert. By using this cDNA as a probe, two additional overlapping clones were isolated: one from the same library and one from a 13-day mouse embryo cDNA library (Fig. 1A). The full-length cDNA (5,742 bp) contained an open reading frame of 1,365 nucleotides beginning at a Kozak (30) consensus sequence (GGAGT$\underline{\text{ATG}}$G); another in-frame ATG resides 21 nucleotides before the Kozak site (Fig. 1B). The coding region was flanked by a 5′ untranslated region of 1,392 bp and a 3′ untranslated region of 2,988 bp containing a poly(A)$^+$ tail (Fig. 1B). Approximately 1,260 nucleotides in the open reading frame (1473 to 2733) are 65 and 70% identical to those of p85β and p85α, respectively; the extreme 5′ coding region and the untranslated regions were unrelated to p85.

The conceptual translation reveals a 55-kDa protein (455 to 462 residues, depending on the start site) that contains two SH2 domains (Fig. 2). The NH_2-terminal SH2 (nSH2) and COOH-terminal SH2 domains are 89 and 81% identical to p85α, respectively, and 83 and 74% identical to p85β, respectively (21, 28). The region between the SH2 domains is also similar to p85, including 35 amino acids that correspond to the minimal p110 binding site in p85α (11). On the basis of these characteristics, we designated this protein p55^{PIK}.

The SH3 domain and bcr homology region found in p85 are replaced in p55^{PIK} by a unique 30-residue NH_2 terminus followed by a conserved proline-rich motif (24). This region contains a putative tyrosine phosphorylation site in a YXXM motif, which if phosphorylated may bind to the SH2 domains. Thus, p55^{PIK} is expected to bind p110 and play a novel regulatory role during stimulation of cells with insulin or IGF-1 and other growth factors or cytokines.

Expression of p55^{PIK} mRNA in mouse tissues. Northern analysis of poly(A)$^+$ RNA with a p55^{PIK} cDNA probe revealed a single mRNA species of 5.7 kb in various mouse tissues (Fig. 3A). The mRNA was distinct from those of p85α and p85β, which migrated slightly above and below this band, respectively (data not shown). Moreover, oligonucleotide probes based on 5′ and 3′ untranslated regions of p55^{PIK} revealed the same 5.7-kb molecule, suggesting that the full-length cDNA corresponds to this message (data not shown). Northern analysis of total RNA in various tissues of the adult mouse demonstrated that the level of p55^{PIK} mRNA expression was highest in the brain and testes; however, it was also detected in adipose, kidney, heart, and lung tissues and skeletal muscle but barely detected in the liver and spleen (Fig. 3B).

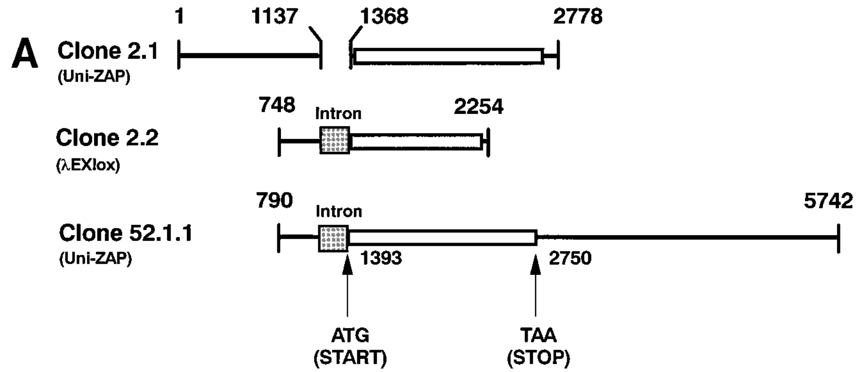
The levels of p85α, p85β, and p55^{PIK} mRNA in various mouse tissues were measured during fetal and postnatal development. The p55^{PIK} was expressed mainly in the brains of 13- and 17-day embryos; however, it was also detected in the legs and viscera. The mRNAs for both p85α and p85β were also detected in these tissues (Fig. 3C). Four days after birth, the levels of p55^{PIK} in brain and kidney tissues, were most abundant, and the levels in heart and lung tissues were significant. By comparison, p85α and p85β were most abundant in the brain and present in all other tissues examined (Fig. 3C). Twenty-six days later, p55^{PIK} mRNA was most abundant in the brain and testes; p85α and p85β were also relatively abundant in the brain. All three messages remained detectable in heart, adipose, kidney, lung, and muscle tissues; however, the level of p85α in the testes was very low (Fig. 3C).

Association of p55^{PIK} with p110 in mouse tissues. To examine the biochemical and functional properties of p55^{PIK}, polyclonal antibodies specific for p55^{PIK} (αp55^{NT}) were prepared. To compare the levels of expression of p55^{PIK} in various mouse tissues, αp55^{NT} was used in conjunction with antibodies against a common region in p85α, p85β, and p55^{PIK} (αp85^{PAN}) or the catalytic subunit of PI-3 kinase (αp110). Immunoprecipitation and immunoblotting with αp85^{PAN} revealed several proteins in tissue extracts from mouse livers, testes, brains, and adipose tissue, including four prominent bands of 85, 80, 55, and 45 kDa (Fig. 4). The 85-kDa protein was also immunoprecipitated with αp110, indicating that it was p85; however, the 80-kDa protein was not immunoprecipitated with αp110, and its identity remains unknown (Fig. 4). The 55-kDa protein was immunoprecipitated with both αp110 and αp55^{NT}, suggesting that it was p55^{PIK} (Fig. 4). Consistent with the Northern blots, p55^{PIK} was most abundant in the brain and testes, detectable in adipose tissue, and nearly absent from the liver. The 45-kDa protein was precipitated from all four tissues with αp85^{PAN} and αp110 but not αp55^{NT} (Fig. 4). This protein may be an NH_2-terminally truncated p55^{PIK} which no longer contains the epitope recognized by αp55^{NT}, an alternately spliced form of p85, or a unique protein related to p85.

Function of p55^{PIK} in transfected CHO cells. CHO cells do not contain significant amounts of p55^{PIK}, as assessed by immunoblotting with αp55^{NT} and αp85^{PAN} (data not shown). Thus, transfected CHO cells were used to investigate the relationship between p55^{PIK}, the insulin receptor, and endogenous IRS-1. CHO cells or CHO^{IR} cells were transfected with p55^{PIK} which contained a FLAG tag at the COOH terminus. CHO^{IR}/p55^{PIK} cells were stimulated with insulin, and extracts were immunoprecipitated with αp55^{CFT} and immunoblotted with αPY. Before insulin stimulation, p55^{PIK} was tyrosine phosphorylated, and this phosphorylation was stimulated significantly by insulin (Fig. 5A). IRβ and IRS-1 were also detected in the αp55^{CFT} immunoprecipitate from the insulin-stimulated cells (Fig. 5A). Moreover, p55^{PIK} was also immunoprecipitated from insulin-stimulated CHO^{IR}/p55^{PIK} cells with antibodies against IRS-1 (Fig. 5B). Thus, p55^{PIK}, as shown previously for p85, associated with IRS-1 during insulin stimulation; however, unlike p85, p55^{PIK} was also tyrosine phosphorylated. The presence of the insulin receptor in the p55^{PIK} immunoprecipitates likely represents the association between IRS-1 and the receptor, as previously shown (5).

The regulation of PI-3 kinase activity associated with p55^{PIK} in CHO cells transfected with the insulin receptor, p55^{PIK}, or both was studied (Fig. 5C). Before expression of p55^{PIK}, insulin had no effect on PI-3 kinase activity in αp55^{CFT} immunoprecipitates from CHO or CHO^{IR} cells, which is consistent with the absence of p55^{PIK} in these cells (Fig. 5C). However, following transfection of CHO and CHO^{IR} cells with p55^{PIK}, the basal activity of PI-3 kinase in αp55^{CFT} immunoprecipitates was significantly increased (Fig. 5C). Insulin stimulated the PI-3 kinase activity in both cell lines, but the response to insulin was most sensitive in the CHO^{IR}/p55^{PIK} cells (Fig. 5C). Thus, PI-3 kinase associates with p55^{PIK} in CHO cells, and insulin stimulated its activity.

Tyrosine phosphorylation of p55^{PIK} by the insulin receptor. The site of insulin-stimulated tyrosine phosphorylation in p55^{PIK} was identified by manual Edman degradation of various proteolytic fragments from [^{32}P]phosphorylate-labeled p55^{PIK} (14). For sequence analysis, p55^{PIK} was immunoprecipitated from CHO/p55^{PIK} cells and phosphorylated in vitro with the purified insulin receptor and [^{32}P]ATP (Fig. 6A). After trypsin



B

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   1  AATTCGGCACGAGCCAACCCCAAGCACACTTCTGCCTGCTACTGTCTCTGAGCCCAGCAC    60
  61  TACCTCAGTGCCTGTAGAATCATCACTGTTGTCTGGAGTTGTCAGGTCTACGAGGCCGCC   120
 121  TCCTACCCAGCCTGTGGTCCCCAGGCCCCACTTGGACCCAAACTGGAACTCACACAACAG   180
 181  TCCTGTTCCCAGAGGATGAGGACGATGTATTGTTATTGCCACTGGCTGAGCCAGAAGTCC   240
 241  TGGGTTGGTGGAGGCAGAGGATGAACCATTGCTTGCCTGAGGGACTCTAGGCACAATGGT   300
 301  ATGATCCTTGGAAGTGGTTCAGCTTCCCCACTGATCTATGTAGCACAGAAAGTTAGTGTC   360
 361  TCTCCAGCAGGGAGAGCATTCACTGTACACACCTTCTGGTATGACAAGAGACTTCACCAC   420
 421  CACCCTCTGCCCAGCCTGCTATCCAGGTGGCTGTGTTACAAAAGGTCCCACAAGGGTCAC   480
 481  AGGCTTGGACACTCCGTTCTTTCCAGCTCCCCACCCAAAAGTGGCCTTAACTACTAGACT   540
 541  GGTTGGCTGGAGACCTAGCCACCAAGGGGAGGATTTGGCCAGGGTGATGGTTTTGCCAGC   600
 601  CTCCATCCCTGTGGTGCCTGGCCACTTAGAGGACACAAGTGCTTTTGCCCTGTAGCTGCA   660
 661  TCATTGTCCAGGAAATCAAGATGACAAAAATAAAGGAATCATAAACTTCAGCCCTTGGTA   720
 721  AAAAAAAACGGCACGAGGTTACCTTTACTACTCGGGCACAGTCGAGCTGACTGGACTCTC   780
 781  CAAGGGGTCGCCATCCCGAAGCTGCGGAAGCTTGGGTTCCCAGCCTGACGCGAATAGAGC   840
 841  CCGAGTCCCAATCCCAGAGGGAATCGCTCTCCGCAGACCAGTGGGACCCCGAAACTTGAA   900
 901  CGCAAACCCCCTTTTCAAGCCTTTGTCACTTCCCCAGCTTTCTCCCAACGCGTTCTTTTT   960
 961  TCCCCCTTCCTCTCCATTCTCTCTTCTCCGAAGGACACAAAAGTGGCTTCCGCTGAAAGA  1020
1021  TTAGGAGGCGGTGGGAGCTTTTCCCTTTGGAGAGCGATTGTGTAGGAAGGATTTTCGGGA  1080
1081  AGCTGCTTTTTAACACCACTGCTCTTTGCTTTCCGAGCTTCCCTGTAACCCTCTGAGGTA  1140
1141  AAAACCCCGTAGCTTGAAAGTTCGGGGTATTTTGTTGGGTGCTTTAGGAGGAGAGAAGAG  1200
1201  GAGGACCTTGTCCTCATCCTAGTAGTTTGGCTGGACTTGTACTGGCCGTTGGAAACCCCC  1260
1261  AAGTACATTTCCGTGTGGAACTTTTGCAAATATATATTTAGATTTTAAATATCAGATAAA  1320
1321  AGATATATATGCTTTTTATATATTTCCCGACGACCTGCCCCTGACAGCGCGATGTACAAT  1380
                                                        m  y  n   3
1381  ACGGTGTGGAGTATGGACCGCGATGACGCAGACTGGAGGGAGGTGATGATGCCCTATTCG  1440
      t  v  w  s  M  D  R  D  D  A  D  W  R  E  V  M  M  P  Y  S  23
1441  ACAGAACTGATATTTTATATTGAAATGGATCCTCCAGCTCTTCCACCAAAGCCACCTAAG  1500
      T  E  L  I  F  Y  I  E  M  D  P  P  A  L  P  P  K  P  P  K  43
1501  CCAATGACTCCAGCAGTCACAAATGGAATGAAGGACAGTTTCATTTCTCTTCAAGATGCA  1560
      P  M  T  P  A  V  T  N  G  M  K  D  S  F  I  S  L  Q  D  A  63
1561  GAGTGGTACTGGGGAGATATTTCCAGGGAAGAGGTAAATGACAAATTGCGGGACATGCCA  1620
      E  W  Y  W  G  D  I  S  R  E  E  V  N  D  K  L  R  D  M  P  83
1621  GATGGTACCTTCTTAGTTCGTGATGCCTCAACGAAAATGCAGGGGGATTATACATTGACT  1680
      D  G  T  F  L  V  R  D  A  S  T  K  M  Q  G  D  Y  T  L  T  103
1681  TTGAGGAAGGGAGGAAATAATAAATTAATAAAGATCTATCATCGGGATGGTAAATATGGC  1740
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1741  TTCTCTGAGCCCCTGACGTTTACTTCTGTGGTGGAGCTTATTAACCACTACCACCACGAG  1800
      F  S  E  P  L  T  F  T  S  V  V  E  L  I  N  H  Y  H  H  E  143
1801  TCTCTCGCTCAGTACAATCCCAAACTCGACGTGAAGCTGACGTACCCAGTATCCAGATTC  1860
      S  L  A  Q  Y  N  P  K  L  D  V  K  L  T  Y  P  V  S  R  F  163
1861  CAACAGGATCAGTTGGTAAAAGAAGATAACATTGATGCAGTAGGTAAAAATCTGCAGGAG  1920
      Q  Q  D  Q  L  V  K  E  D  N  I  D  A  V  G  K  N  L  Q  E  183
1921  TTCCACTCTCAGTATCAGGAGAAGAGCAAAGAGTATGACAGGCTGTATGAAGAGTACACA  1980
      F  H  S  Q  Y  Q  E  K  S  K  E  Y  D  R  L  Y  E  E  Y  T  203
1981  AGGACATCACAGGAAATACAAATGAAGAGGACTGCCATTGAAGCCTTTAATGAAACAATT  2040
      R  T  S  Q  E  I  Q  M  K  R  T  A  I  E  A  F  N  E  T  I  223
2041  AAAATATTTGAGGAGCAGTGTCATACCCAAGAACAACACAGTAAAGACTATATCGAGCGC  2100
      K  I  F  E  E  Q  C  H  T  Q  E  Q  H  S  K  D  Y  I  E  R  243
2101  TTTCGCAGAGAGGGGAATGAGAAGGAGATCGAGCGAATTATGATGAATTATGATAAATTG  2160
      F  R  R  E  G  N  E  K  E  I  E  R  I  M  M  N  Y  D  K  L  263
2161  AAATCACGTCTTGGTGAGATTCATGATAGCAAACTGCGTCTTGAGCAGGACTTGAAGAAA  2220
      K  S  R  L  G  E  I  H  D  S  K  L  R  L  E  Q  D  L  K  K  283
2221  CAAGCTTTGGACAACCGGGAAATAGATAAAAAAATGAATAGCATCAAACCCGACTTGATC  2280
      Q  A  L  D  N  R  E  I  D  K  K  M  N  S  I  K  P  D  L  I  303
2281  CAGCTGCGTAAGATCCGGGATCAGCACCTTGTATGGCTCAATCACAGAGGAGTGAGGCAG  2340
      Q  L  R  K  I  R  D  Q  H  L  V  W  L  N  H  R  G  V  R  Q  323
2341  AGGCGCCTGAATGCCTGGCTGGGGATCAAGAATGAGGACTCAGATGAAAGCTATTTTATC  2400
      R  R  L  N  A  W  L  G  I  K  N  E  D  S  D  E  S  Y  F  I  343
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2401  AATGAGGAAGATGAGAACCTGCCGCATTATGATGAGAAAACCTGGTTTGTGGAGGATATC  2460
      N  E  E  D  E  N  L  P  H  Y  D  E  K  T  W  F  V  E  D  I   363
2461  AACCGAGTACAAGCAGAGGACTTGCTTTATGGGAAACCAGATGGTGCATTCTTAATTCGT  2520
      N  R  V  Q  A  E  D  L  L  Y  G  K  P  D  G  A  F  L  I  R   383
2521  GAGAGTAGCAAGAAAGGATGTTACGCTTGTTCTGTGGTTGCAGACGGGGAAGTGAAGCAC  2580
      E  S  S  K  K  G  C  Y  A  C  S  V  V  A  D  G  E  V  K  H   403
2581  TGTGTCATCTACAGCACGGCTCGAGGATATGGCTTTGCAGAACCCTACAACCTGTACAGC  2640
      C  V  I  Y  S  T  A  R  G  Y  G  F  A  E  P  Y  N  L  Y  S   423
2641  TCACTGAAGGAGCTGGTGCTCCATTACCAGCAGACATCCCTGGTTCAGCACAACGACTCC  2700
      S  L  K  E  L  V  L  H  Y  Q  Q  T  S  L  V  Q  H  N  D  S   443
2701  CTCAACGTCAGGCTCGCCTACCCTGTCCATGCACAGATGCCTACGCTCTGCAGATAAGCA  2760
      L  N  V  R  L  A  Y  P  V  H  A  Q  M  P  T  L  C  R  *     461
2761  GAGTGGAAGAGACACACTCTCTAGCCGTTTTTTTCCTATGGTTTTTATTAGACTACGATG  2820
2821  AGGGCATTCTTTCAACGTAGACTGCTTGTTTGCACAAGTGATTCTGTGAATGTGAAATGG  2880
2881  AGAGGCCAAGCAGTAGCTTGGATTTAGAAATGAGGGGCCCAGGGTCTCTGGCCTCGGCTG  2940
2941  TGCTGCTGCACTGATGGACTAAGCTGGAAGCAGATATTGGTTTCATGGGGTTTGGGTTTG  3000
3001  TTGTCAGGCACCTTTAAAAGAACAGCTAAGGCTTGTTGTGGGTTGGGGTGGGGGTTTTAT  3060
3061  TTGGAAGTTTCTGAAGAGTCCACATCCCTTTGTCCTCAACCCTAAGAATGCAGCAGGTCA  3120
3121  CAGTTCTGCTGGGAGTTGTTTTGATTTGATAGTCTCTTCCCCTTTCCCCCAAATAAAGAG  3180
3181  CCGATTTTGGCTCTGTGGTAAAGTGGGATTTGGTTTGGAGGGAAAAACAACCAAAGGAAA  3240
3241  ATAGGGAGGTATGGGATTACATTTTCAGAATCTAAACCAAGGAGGCAAAAGACCCCTTCA  3300
3301  GTTGATGTTACTTCAATTTTATCAACATAATCTAGGCTTCAGCATCTTCACCAACTCCTC  3360
3361  CCTCTAAAGCACTGTGTTCAAAAACCAACAAAGCAGCATCGCCNAGACCAAGGTCTAAGG  3420
3421  GGAGGACAGTAGTAGCTGAATGTACACTTCTGTACCAAAACTTGAAAGACTAGAAATGTG  3480
3481  AGTTTCAACAAACACTAAAATTGGTCAGTGTATTTCCTTTTGCCCTGGCCTTGTTTCTCA  3540
3541  GATGAGGAATAGAATTATTTTGTGGAAATAGTAAGCTTTGAGTCATAATGAAGTTGGTGC  3600
3601  TTGTGTGGTGTTTCTTTAAAGAAATGTTTGAAACCCTTGTAAGTTGTTTTATGAGTAAAG  3660
3661  AAACAGTGCAATCCAGTGCTTTTAGATGGCTTGATATACCAAATAATGATAGAGAACAAC  3720
3721  ATTGTTGTGTGCTTCCTCAAGTTTAAAAGCCTTGCCAAAACTATACAAGGATTAATTTGC  3780
3781  CTTCATCTCCCCTTCCTTTTTTGGATAGGGTTTAGGGAGCCATAGGTAGCTAAAGGAGGA  3840
3841  CTCGAGTTTGTGGTCAGAGACCTCAGTAAATCACAGGCACATGAGGCCTGGTATCCATGG  3900
3901  TGAAGGGTCCATCACATGACATGTTATTCAATACTGTGGTTGAAGCGTTTGCCAGAAGAG  3960
3961  GGGATGACGTGGAGTTCAGACTATCTGGGGAAATAATCCACAGGCTTTCCTGCTTGCCCT  4020
4021  TTTTGTGAGCCTGCTGTTAAGGCAGTGCACACAGCCTGCTCTCATGCTTCCGTGGCTGTG  4080
4081  GTTTAAGCCTTCAGCTAAGTGAAGTTAGATAGAGGAGAGGGCAGCCATCTATTTATGGAT  4140
4141  TCACACTCATTTAAGAGTTCAGCTGCTTCAGAGTCAGTTCTGGAGCCATAACAGGCTCAG  4200
4201  TATGACTCAGCTGCTTGAGCCCAGTAATGTGCAGTCAGGCAGTTTAGACAAGCAGCCTGT  4260
4261  GCCTGGGTCATCAGGCTTACAATCAGGGAAGATGAAGTTTGGGGGCCAAAATAAAGATGA  4320
4321  ATATGACTTTCCCTGAGCACTTCCTTTGGTGACAGTGTCTAGAAGAAACCACAGTATAGA  4380
4381  GATAGGTCAAAAGTTTTGAATAATTGTCACAGTTGATAGGGCATGCCATTGATGGCTTTT  4440
4441  TCTTGTTCATGCTCCAGTGTGAAAGAGAGGAGATTGACCACCCTCAGCCACTCTGTAAGG  4500
4501  CCTTTTTCAAAATTGCCAGCTTAAAATCTTGCTCAGTTTACCAAGTAATGCCAGGCTATT  4560
4561  TGTTGATTGGAATACCTGTGACTTTGTACTGATGTTGAACTTGCTGAAGCAGTTATATGC  4620
4621  TCAAGATTAGGTGTGAGGAATCCCTCTGATCCAGCACTAAAATTTTAGTATGTCCTGAAC  4680
4681  GCCTTTTTAAAGAAATCTCTTCCAAGTAAGTCAAAATGATAAAATATACAGCTTTAGTGT  4740
4741  TGAATAATGTCTTTACCTTGTAGGCAGACATGGAAGATATGCAGGAGAAAGCAGCATCTA  4800
4801  CACCTGGGCTGGACAATGGAGAAAGACAGGTTTTCAGTCCTATATTCTTTCCCTTTGAGT  4860
4861  AACCACTTTGTGGGAGCTGAGACCAGGGATCATTTAATAAATCGGAAGCTATCTTTTTAT  4920
4921  TTTTCTGCCAAGTTACTACATGATTTATCTGATCCTGAGCTGTGGAAATGGCATGAGGAG  4980
4981  CAGTCTCCTAAGAGTGGCCCTGCTGTCTGAGGGAGTCTGGAAGCAGGTGTTGGTCTTCTT  5040
5041  CTCAAGGCTAGCTCAAAGTTCTGTCTCATGATCTAGGCCCTGGGACTATCTCTTTTGGCA  5100
5101  TCTTAACTGTAGACTCATTGACTAAAGCAGAGGCTAGAGACAGATTAGGACCATAGGGGC  5160
5161  AGGCAGATCAGCCAGTCCCCAGATCAGCCAGTCCCCAACAGGAAAGCAGCTTTGGGTTGG  5220
5221  CTAGATACAGTTTTTAAAATAAAAACAAAACAAAACAAAGCTGTTTCCACCTGGCATAGT  5280
5281  TCAGCTTAGGTAGGTTGTTTATGTTCTTGTCACTGCTCCAGCAATAGATGAAGACATCCT  5340
5341  ACAGCTCCACACTACTAAGACACAAGCTCTCTACATTTACTTCAGACTCAAGCCCGAGTG  5400
5401  GCATCTTCCTTGTGTCCCTTCTCTGCAAGGTACCAGCTTCACCCATTCTCCAGAACTTTA  5460
5461  AAGAAAAAATGTACTTGAACAATTTCTGATTTCTAGGATGATCTCTACTGCCAGTTAGAT  5520
5521  CTTCTTGAGGTTTCCATGACATCATACACCAGAGGTCCATTCTTGGTCCTTTGCTGCCAA  5580
5581  CTGCTCATTCTTGACTTAGCTCTAGCCATTTGTGACAACCACCCTTGTTTCCTTACAAAT  5640
5641  CCTCGCATGTAACTTTGGTACTTTGTTGTTTCTTGTGAAGAATCTATTCTGTTGTCTTTG  5700
5701  ATGTAATAAAAAAATTTCATGTAAAAAAAAAAACTCGTGCCG                    5742
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FIG. 1. cDNA sequence of p55^{PIK}. (A) Clone 52.1.1, containing p55^{PIK} cDNA, was obtained by expression screening with ^{32}P-IRS-1. Clones 2.1 and 2.2 were obtained by hybridization screening with ^{32}P-labeled clone 52.1.1 cDNA probe. The relative position of the open reading frame is shown between the start and stop codons, and a putative intron is highlighted (shaded box). (B) cDNA and deduced amino acid sequences of p55^{PIK}. Nucleotides and amino acids are numbered on the sides of the figure. The putative Kozak start site (underlined boldface), the putative 7-amino-acid NH_2-terminal extension (lowercase), and the stop codon (*) are indicated. The calculated molecular weight of p55^{PIK} beginning at the Kozak site is 53,600; with the 7-amino-acid extension, the calculated weight is 54,481.

digestion, a single phosphopeptide was resolved by Tricine-SDS-PAGE (Fig. 6C). This tryptic peptide was analyzed by Edman degradation or further digested with endoproteinase Glu-C (V8 protease) or endoproteinase Asp-N before Edman degradation. Radioactive phosphate was released from the original tryptic peptide at cycle 8, whereas radioactivity was released from the endoproteinase Glu-C and Asp-N fragments at cycles 2 and 4, respectively (Fig. 6D). This pattern of ^{32}P-Tyr

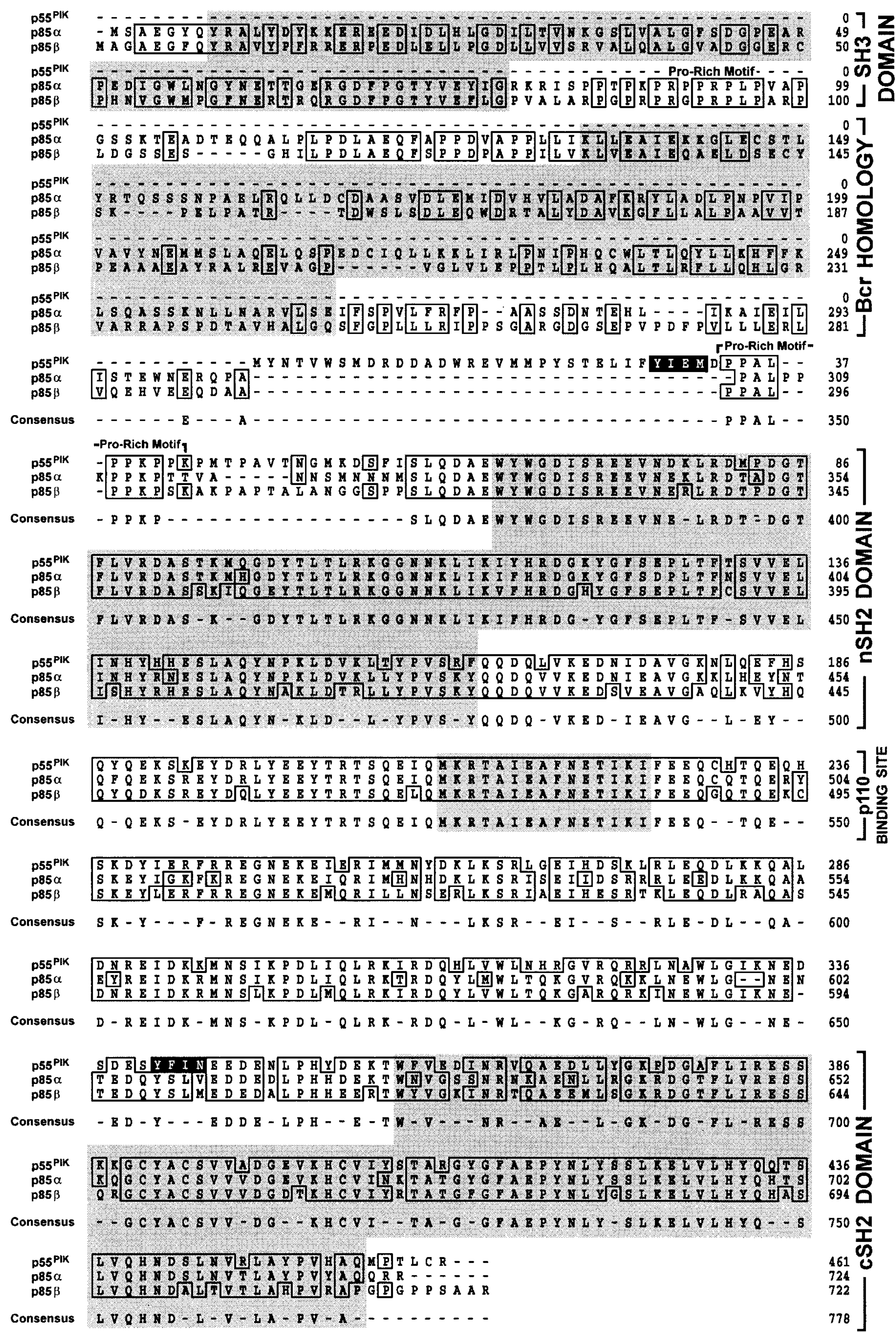

FIG. 2. Alignment of amino acid sequences of p55^{PIK}, p85α, and p85β. The amino acid residues for each peptide, with the addition of gaps (−) to optimize the alignment, are numbered to the right of each sequence. Similar residues are boxed, and the identities are shown below. Recognized domains are shaded, including the SH3 domains, the bcr homology region, the nSH2 domain, the p110 binding site, and the COOH-terminal SH2 domains (cSH2); the proline-rich motif is indicated. Putative tyrosine phosphorylation sites in p55^{PIK} (Y-341 and Y-29) are in black boxes.

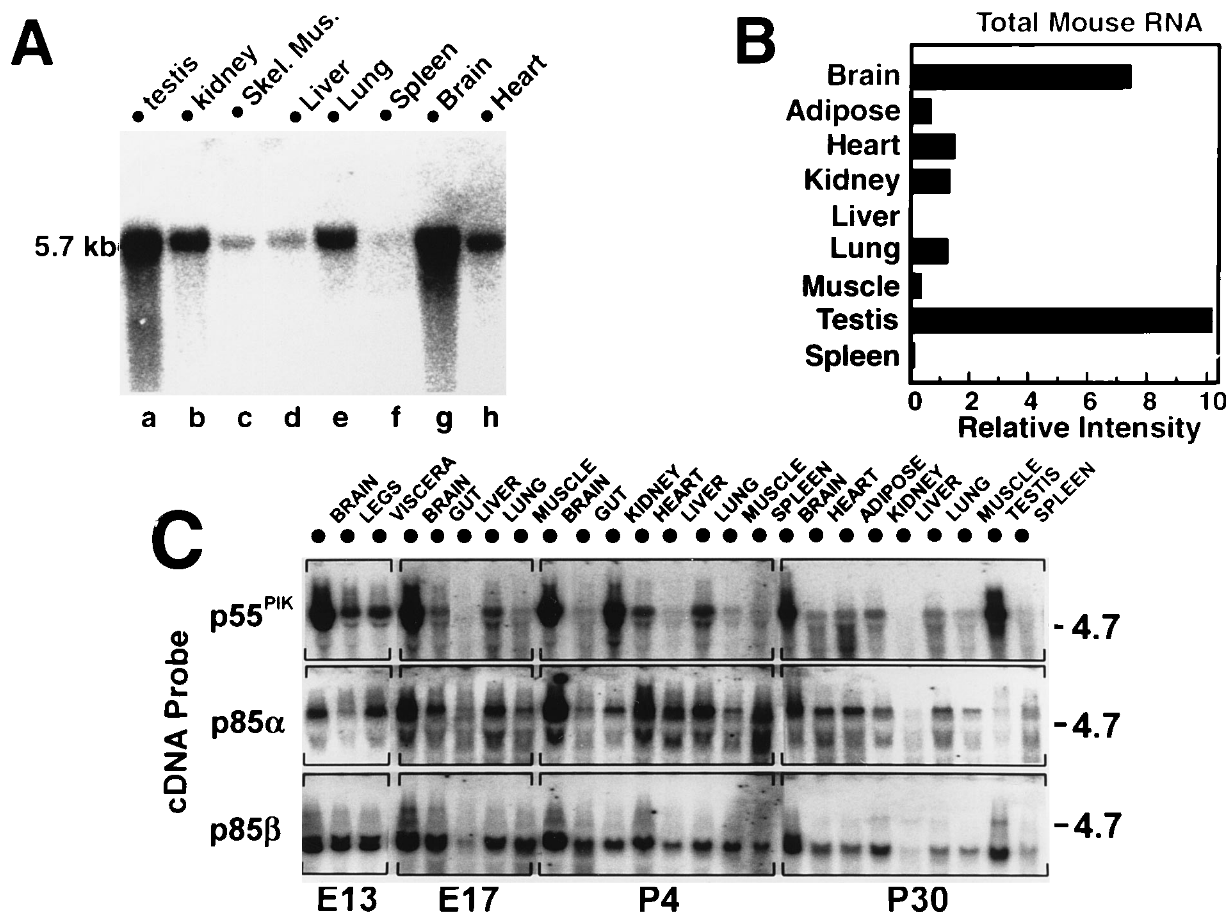

FIG. 3. Expression of p55^{PIK} in tissues. (A) Poly(A)$^+$ mRNAs from various mouse tissues were hybridized with a ^{32}P-cDNA probe encoding nucleotides 790 to 5742 of p55^{PIK} as described in Materials and Methods. Skel. Mus., skeletal muscle. (B) Total RNA (20 μg) isolated from a variety of tissues from 4-week-old male mice was probed as described above. The graph represents the p55^{PIK} mRNA levels in each tissue. Detection and quantification were done with a Phosphorimager and Imagequant (Molecular Dynamics). (C) By using total RNA isolated from the indicated embryonic (E13 and E17, embryonic days 13 and 17, respectively) and postnatal (P4 and P30, postnatal days 4 and 30, respectively) stages of mouse development, three identical filters containing 20 μg of RNA per lane were prepared. Equal loading and transfer were checked by ethidium bromide staining. The filters were hybridized with p55^{PIK}, p85α, or p85β cDNA probes as described in Materials and Methods. The radioactivity was detected with a Phosphorimager. The position of the 28S rRNA (4.7 kb) is indicated on each blot.

release unambiguously identified Tyr-341 in p55^{PIK} as a major in vitro phosphorylation site. Phosphorylation of Tyr-29 in the YXXM motif was not detected.

To verify phosphorylation of Tyr-341 in vivo, p55^{PIK} was immunoprecipitated from ^{32}P-labeled CHO^{IR}/p55^{PIK} cells. Before insulin stimulation, p55^{PIK} was heavily serine phosphorylated, and the increased phosphorylation due to insulin was revealed as a slightly reduced rate of migration during SDS-PAGE (Fig. 6B). Multiple tryptic phosphopeptides were obtained from p55^{PIK} labeled in vivo before and after insulin stimulation. The major insulin-stimulated peptide comigrated with the single Tyr-341-containing peptide obtained during in vitro labeling (Fig. 6C); phosphoamino acid analysis revealed only phosphotyrosine (data not shown). Thus, Tyr-341, located in a YFIN motif, is a major phosphorylation site in p55^{PIK} during insulin stimulation of CHO^{IR}/p55^{PIK} cells.

Selectivity of the NH$_2$-terminal SH2 domain in p55^{PIK}. Previous work showed that both SH2 domains in p85 preferentially bind to several phosphotyrosine residues in IRS-1 (Tyr-460, Tyr-608, Tyr-987, and Tyr-939) which are located within YMXM motifs (56). To compare the selectivity of p55^{PIK}, the binding activities of GST fusion proteins containing the nSH2 domains of p55^{PIK} and p85β were compared. Tryptic phosphopeptides from ^{32}P-IRS-1 were incubated with 1 or 10 μg of the GST-nSH2 fusion proteins (57). At the lowest concentration of fusion protein, a tryptic peptide containing the phosphorylated Y-939MNM motif preferentially bound to both fusion proteins (Fig. 7). At the higher concentration, phosphopeptides containing Y-608MPM and Y-987MTM also bound to both nSH2 domains; a fourth peptide containing the Y-460ICM motif bound only to the nSH2 domain of p55^{PIK} (Fig. 7). These results suggest that the nSH2 domains of p55^{PIK} have similar but perhaps not identical selectivities for binding to the nSH2 domains of p85β and, as determined by previous studies, to p85α; the COOH-terminal SH2 domain of p55^{PIK} was not studied, as it is expected to be identical to that of the p85 isoforms (56).

Regulation of PI-3 kinase by association with IRS-1. The regulation of PI-3 kinase by tyrosine phosphorylation of p55^{PIK} or its association with IRS-1 was investigated with Sf9 cells. To establish that p55^{PIK} was tyrosine phosphorylated by IRβ in Sf9 cells, either p55^{PIK} (including the COOH-terminal FLAG tag) or p85α was coinfected with IRβ by using the appropriate recombinant baculovirus (1). Sf9^{IRβ} cell lysates immunoprecipitated with αp85^{PAN} and immunoblotted with this antibody showed that the two proteins were expressed at approximately

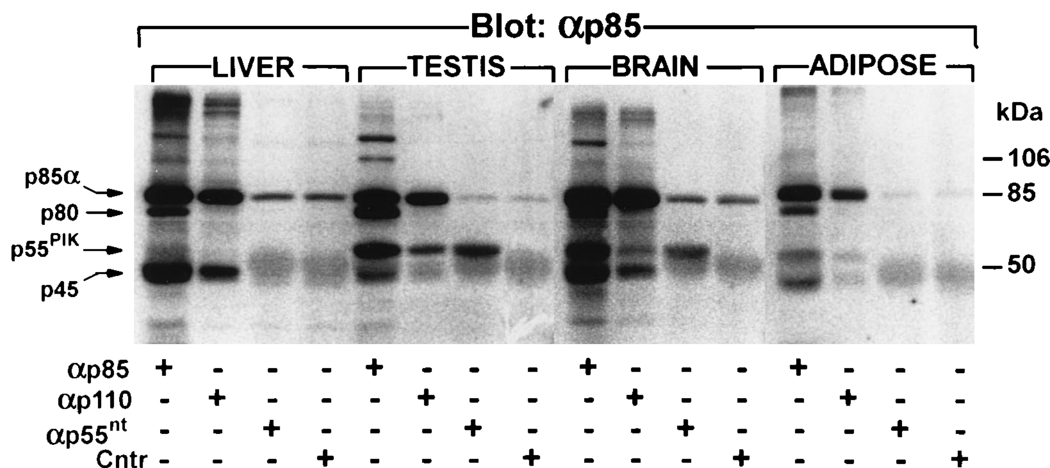

FIG. 4. Identification of p55^{PIK} in mouse tissues. Clarified lysates from various mouse tissues were immunoprecipitated with αp85^{PAN}, αp110, αp55^{NT}, or nonimmune serum (Cntr) as indicated below the immunoblot. The immune complexes were collected with protein A-Sepharose, reduced, separated in an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the αp85^{PAN} antibody. The bands corresponding to p85α and p55^{PIK} are indicated on the left.

equal levels after viral infection (Fig. 8A). Moreover, immunoblotting with αPY revealed that p55^{PIK}, but not p85α, was tyrosine phosphorylated (Fig. 8B). In these experiments, the insulin receptor was not coimmunoprecipitated with p85 or p55^{PIK}, suggesting that these SH2 proteins do not bind directly to the β-subunit under these conditions.

To demonstrate that p55^{PIK} associated with p110 in this system, Sf9 cells were coinfected with p110 and p55^{PIK} and PI-3 kinase activity in αp55^{CFT} and αp110 immunoprecipitates was measured. Equal levels of PI-3 kinase activity were strongly immunoprecipitated with αp110 from Sf9 cells expressing p110 alone or together with p55^{PIK} (Fig. 8C). PI-3 kinase activity was also immunoprecipitated with αp55^{CFT} from the cells coinfected with p55^{PIK} and p110 (Fig. 8C). Thus, p55^{PIK} was tyrosine phosphorylated during expression with IRβ and associated with p110.

To determine whether tyrosine phosphorylation of p55^{PIK} stimulated PI-3 kinase, Sf9 cells expressing both p55^{PIK} and p110 were also infected with IRβ, IRS-1, or both. Expression of IRβ had no effect on the PI-3 kinase activity in αp55^{CFT} immunoprecipitates, suggesting that tyrosine phosphorylation of p55^{PIK} alone was insufficient to activate the kinase (Fig. 8D). Coinfection of rat IRS-1 without IRβ had no effect on PI-3 kinase activity in αp55^{CT} immunoprecipitates; however, expression of IRβ and IRS-1 together with p55^{PIK} and p110 resulted in a twofold stimulation of PI-3 kinase activity (Fig. 8D). Thus, similar to the case for p85, occupancy of the SH2 domain in p55^{PIK} activated p110 (40, 57), whereas tyrosine phosphorylation of p55^{PIK} did not activate PI-3 kinase.

DISCUSSION

IRS-1 is tyrosine phosphorylated during stimulation of responsive cells with insulin or IGF-1, IL-4 or IL-9, growth hormone, or IFN-α (41). IRS-1 contains multiple tyrosine phosphorylation sites in various amino acid sequence motifs which bind various SH2 proteins, including p85α and p85β, Grb-2, nck, SH-PTP2, and c-fyn (4, 35, 39, 41). In the cases of PI-3

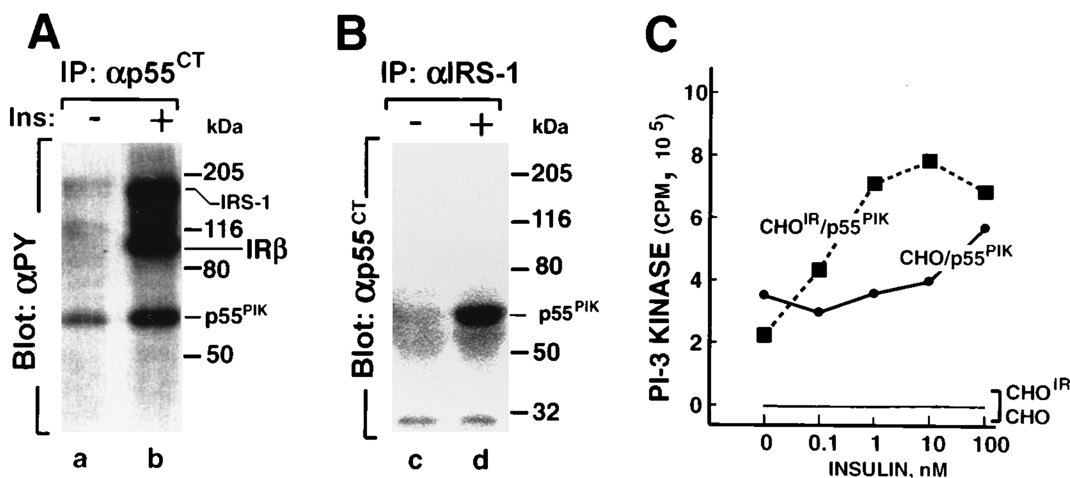

FIG. 5. Phosphorylation of p55^{PIK} CHO cells. (A) CHO^{IR}/p55^{PIK} cells were incubated without or with 100 nM insulin (Ins) for 5 min as indicated above the lanes, and cell extracts were immunoprecipitated (IP) with αp55^{CFT} and immunoblotted with αPY. (B) Extracts equivalent to those prepared for the previous experiments were immunoprecipitated with αIRS-1 and immunoblotted with αp55^{CFT}. (C) PI-3 kinase activity in αp55^{CFT} immunoprecipitates from CHO or CHO^{IR} cells before or after expression of p55^{PIK} was measured. Each point is the average of three determinations, and the standard deviations are <10%.

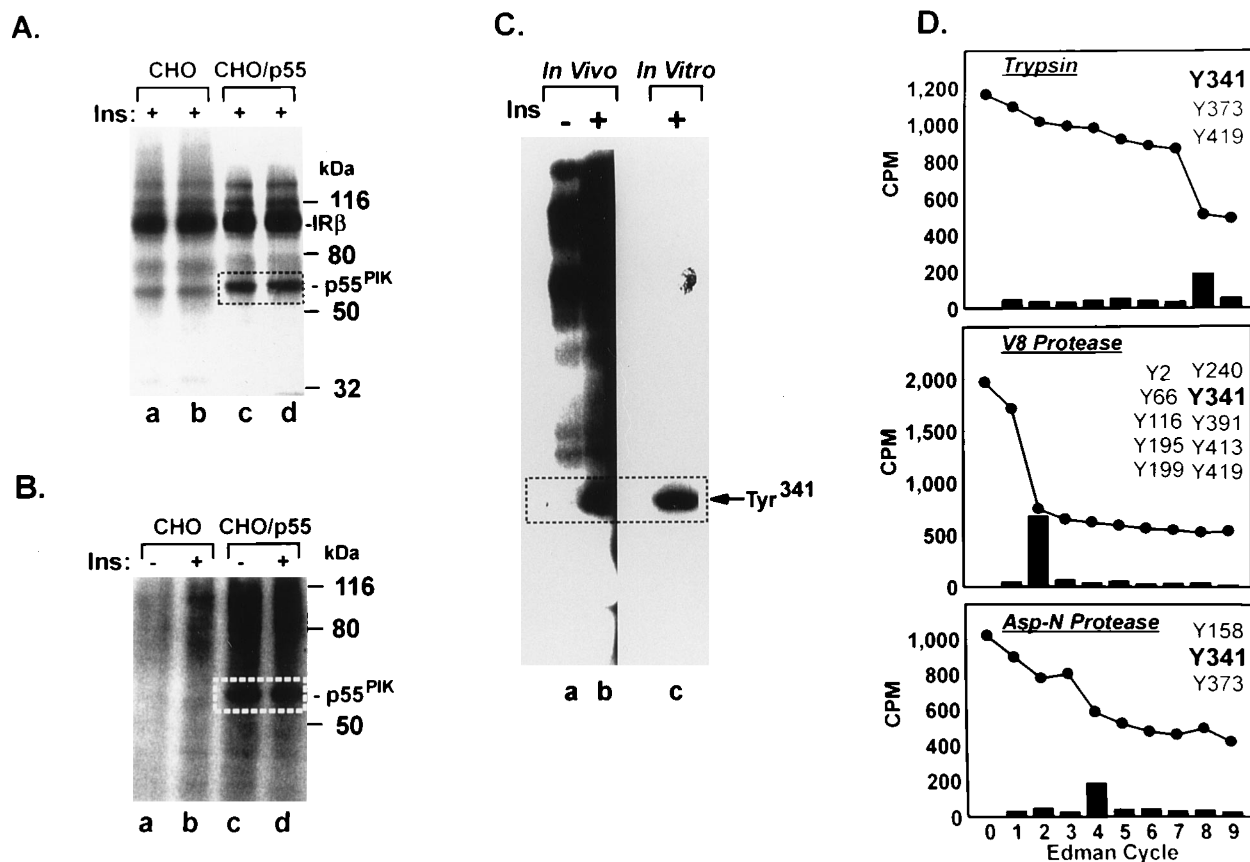

FIG. 6. Identification of the tyrosine phosphorylation site in p55^{PIK}. (A) In vitro phosphorylation. Parental CHO cells and CHO/p55^{PIK} cells were lysed and immunoprecipitated with αp55^{CFT}. The immunoprecipitates were labeled with [^{32}P]ATP by insulin-stimulated, wheat germ agglutinin-purified insulin receptor; resolved by SDS-PAGE; transferred to a PVDF membrane^{PIK} and detected by autoradiography. (B) In vivo phosphorylation. Control CHO^{IR} cells and CHO^{IR}/p55; cells were labeled in vivo with $^{32}P_i$ and incubated in the absence or presence of 100 nM insulin (Ins) for 10 min. Proteins in the cell lysates were immunoprecipitated with αp55^{CFT}, separated by SDS-PAGE, transferred to a PVDF membrane, and detected by autoradiography. (C) Tryptic digest. ^{32}P-p55^{PIK} from panels A and B was excised from the PVDF membrane as indicated (dashed boxes), trypsinized in situ, and separated by Tricine-SDS-PAGE. (D) Edman degradation. The tryptic peptides obtained from in vitro-labeled p55^{PIK} were analyzed by manual radiosequencing before or after a secondary digestion with V8 protease (endoproteinase Glu-C) or endoproteinase Asp-N. The radioactivity released from the disk (bars) and the radioactivity left on the disk following each cycle (solid circles) are shown. Potential tyrosine phosphorylation sites consistent with each result are indicated (the common site is in boldface).

kinase and SH-PTP2, the association with IRS-1 activates the enzymes (4, 34). Thus, phosphorylated IRS-1 provides a common interface between diverse membrane receptors and cytoplasmic SH2 proteins which mediate at least partially the pleiotropic biological response.

In order to identify novel proteins that bind to phosphorylated IRS-1, we modified the CORT technique, which was used previously to clone SH2 proteins that bind to the activated epidermal growth factor receptor (36). Several new proteins that associate with IRS-1 were detected, including p55^{PIK}, which associates with p110 and regulates its PI-3 kinase activity. p55^{PIK} is a unique protein that is homologous to the COOH-terminal portion of p85α and p85β. It contains two similar SH2 domains flanking a region which interacts specifically with the NH_2 terminus of p110 (11). As expected, the SH2 domains of p55^{PIK} bind to the same phosphorylated YMXM motifs in IRS-1 that are selected by p85α and p85β (56). Moreover, during association with phosphorylated IRS-1, the p55^{PIK}-p110 complex is activated. Thus, p55^{PIK} links IRS-1 to p110 in a manner that is similar to that of p85. It is likely that other growth factors or cytokines that stimulate IRS-1 phosphorylation also engage p55^{PIK}. We also expect certain growth factor receptors to engage p55^{PIK} directly through their autophosphorylation sites or other auxiliary subunits.

The p55^{PIK} molecule contains a short NH_2 terminus which lacks the interactive and regulatory regions found at the NH_2 termini of p85α and p85β, including the SH3 domain, homology to the breakpoint cluster region (bcr) gene, and an NH_2-terminal proline-rich motif (11). The proline-rich motif associates with SH3 domains in various proteins, and the bcr homology region may interact with small GTP-binding proteins (24, 46). On the basis of our work with p55^{PIK}, these elements are not necessary for the regulation of PI-3 kinase by IRS-1. However, these elements are thought to link PI-3 kinase to other signaling pathways or structural elements (11); their absence may alter significantly the signaling potential of p55^{PIK}.

Unlike p85α and p85β, p55^{PIK} is tyrosine phosphorylated by the insulin receptor in CHO and Sf9 cells. A major tyrosine phosphorylation site in p55^{PIK} is located in a DESY-34/FINEE motif. The p85α and p85β isoforms each contain a tyrosine residue (Tyr-607 and Tyr-601, respectively) in homologous locations, but the surrounding amino acid sequence motifs are different (EDQYSLVED in p85α and EDQYSLMED in p85β). The p85 isoforms are not readily tyrosine phosphorylated by the insulin receptor (57), although overexpression of the insulin receptor and p85α sometimes results in phosphorylation at multiple sites, including Tyr-607 (17). From our

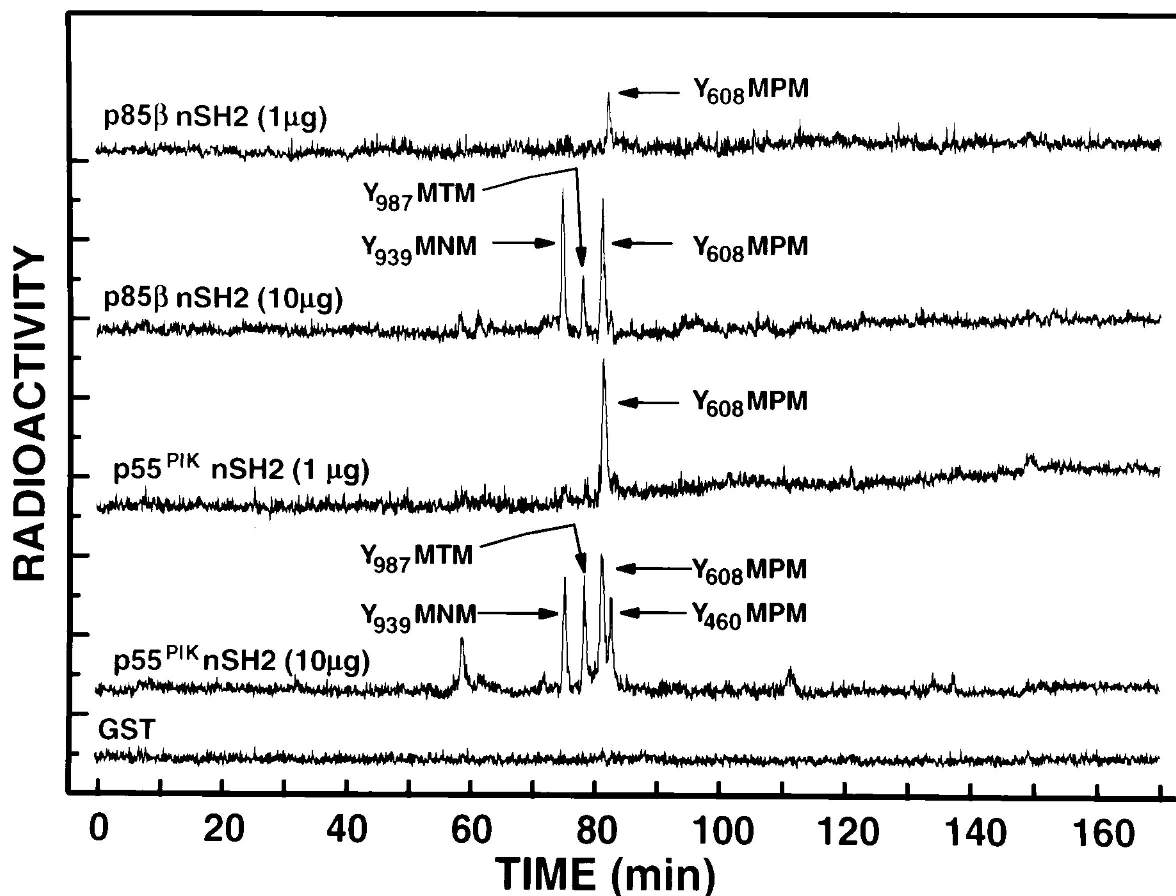

FIG. 7. Binding of tryptic phosphopeptides from ^{32}P-IRS-1 to the nSH2 domains of p85β and p55^{PIK}. The nSH2 domains of p55^{PIK} or p85β were expressed as bacterial GST fusion proteins. Recombinant IRS-1 purified from Sf9 cells was labeled in vitro with purified insulin receptor, digested with trypsin, and incubated with two concentrations of GST-SH2 fusion proteins. Bound peptides were separated by reversed-phase high-pressure liquid chromatography and identified by radiosequencing and comparison with previous results (57).

results with Sf9 cells, the insulin receptor apparently recognizes and phosphorylates p55^{PIK} directly but does not form a stable complex with it. Tyrosine phosphorylation of p85 has been variably reported for other systems as well (20, 25, 47). The serine residue next to the tyrosine in p85 is phosphorylated by p110 (12); however, in p55^{PIK}, the serine precedes Tyr-341, and Ser-340 does not appear to be phosphorylated.

The function of tyrosine phosphorylation in p55^{PIK} is unknown. Tyrosine phosphorylation of p55^{PIK} does not stimulate PI-3 kinase activity in Sf9 cells. It is possible that the phosphorylated YFIN motif in p55^{PIK} provides a regulated binding site that links the PI-3 kinase to unique SH2 proteins that are inaccessible to p85 complexes. In this model, the NH_2-terminal interactive sites found in p85 are not required and could disrupt the specific signaling function of p55^{PIK}. Phosphopeptide libraries have been useful for the identification of phosphotyrosine-containing motifs recognized by various SH2 proteins; however, an SH2 domain specific for the phosphorylated YFIN motif has not been revealed (52).

The unique NH_2 terminus of p55^{PIK} also contains a potential tyrosine phosphorylation site in a YXXM motif. This residue, Tyr-29, is located near negatively charged amino acids, making it a likely phosphorylation site; however, we did not detect its phosphorylation in our experiments. This YXXM motif is particularly interesting as it has the potential to bind to the SH2 domains in p55^{PIK}. This site could constitute an intramolecular regulatory mechanism analogous to that described for Src kinases. Intramolecular association between Tyr-29 and one of the SH2 domains may block activation by heterologous molecules such as IRS-1. By contrast, Tyr-29 may provide an intramolecular mechanism for PI-3 kinase activation. Further studies with mutant molecules will be needed to evaluate these possibilities.

Several reports describe at least two phosphoproteins of approximately 60 kDa that undergo insulin-stimulated tyrosine phosphorylation in various cell types, including rat adipocytes, 3T3-L1 cells, and CHO cells (19, 26, 33, 38, 59, 69). One of these proteins appears to associate with rasGAP (19). The other protein, called pp60, associates with PI-3 kinase (33); in rat adipocytes, this protein was detected at 55 kDa (59). Our p55^{PIK} displays many characteristics in common with pp60 and may be the same protein. Like p55^{PIK}, pp60 is tyrosine phosphorylated in vivo during insulin stimulation and in vitro with the purified insulin receptor. Moreover, immunoprecipitates of p85 from insulin-stimulated adipocytes contain tyrosine-phosphorylated pp60 and IRS-1 (26, 33). Immunofluorescence reveals that p55^{PIK} associates with the plasma membrane of transfected CHO cells during insulin stimulation (data not shown), which is consistent with the recovery of pp60 in plasma membrane fractions of insulin-stimulated adipocytes (26, 33). A careful comparative study will be required to determine

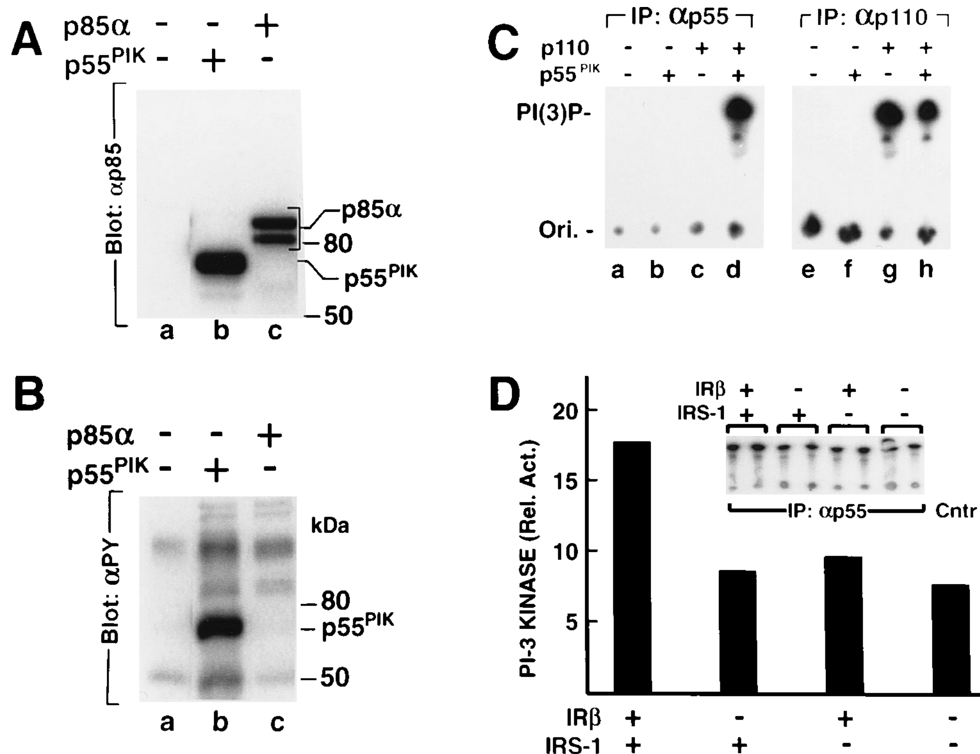

FIG. 8. (A) Sf9 cells were infected with a baculovirus containing IRβ and p55PIK, p85α, or no insert and incubated for 48 h. Cells lysates were immunoprecipitated and immunoblotted with αp85PAN. (B) Sf9 immunoprecipitates from the experiment whose results are shown in panel A were immunoblotted with αPY. (C) Sf9 cells were infected with a baculovirus containing p55PIK, p110, or no insert or doubly infected with p55PIK and p110 and incubated for 48 h. Cell lysates were incubated with αp55CFT or αp110, and the PI-3 kinase activity in the immunoprecipitates (IP) was measured. Ori., origin. (D) Sf9 cells were infected with recombinant baculovirus containing p55PIK and p110 in combination with a baculovirus expressing IRβ, IRS-1, or both. After 48 h, the cells were lysed and the extracts were immunoprecipitated with αp55CFT. The immunoprecipitates were washed and assayed for PI-3 kinase activity. Reaction products were resolved by thin-layer chromatography (inset). Immunoblots (not shown) verified that p55PIK, p110, IRβ, and IRS-1 were expressed to the same level in the appropriate Sf9 cells. This experiment was conducted three times with similar results. Rel. Act., relative activity; Cntr, control.

whether p55PIK is the cDNA clone for pp60, a related isoform, or a distinct protein.

PI-3 kinase is implicated in insulin-stimulated glucose transport. Wortmannin and LY294002 inhibit insulin-stimulated PI-3 kinase and glucose uptake in similar concentration ranges (7, 16, 50). Interestingly, expression of mutant p85 molecules that do not bind p110 inhibits insulin-stimulated glucose uptake in CHO cells (16). Together, these results suggest that PI-3 kinase is an essential, but not necessarily sufficient, upstream regulator of glucose transport. It is possible that p55PIK plays a unique role in this process, especially if phosphorylation of the YFIN motif during insulin stimulation couples to regulatory elements controlling the translocation of glucose transporters. Proteins that associate with the phosphorylation site in p55PIK might undergo serine phosphorylation by p110. However, the level of expression of p55PIK is low in adipocyte tissue and skeletal muscle in comparison with that in brain and testis tissues, so the coupling mechanism must be very sensitive.

In rat PC12 cells, PI-3 kinase is necessary for multiple steps of neurite outgrowth during nerve growth factor-stimulated differentiation (27). p55PIK may contribute to this process in vivo, since it is at its highest level early in embryonic development. The different NH_2-terminal regions of p85α and p85β and p55PIK could contribute to the dramatic changes occurring in the cell morphology during neuronal differentiation. In this model, p55PIK may function in the neuron cell body at the beginning of neurite outgrowth, whereas p85α and p85β, through their SH3 domains, may bind to dynamin to mediate the transport of PI-3 kinase to distal parts of the growing neurites (51). IGF-1 plays an important role in the development of many regions of the brain, and the IGF-1 receptor is very abundant in the early developing brain (60). Thus, p55PIK may operate downstream of IGF-1 receptors during central nervous system development.

In summary, we have identified a new regulatory element for PI-3 kinase that is tyrosine phosphorylated on a novel motif during insulin stimulation; this motif could interact with SH2 proteins. Since p55PIK lacks the NH_2-terminal motifs of p85, it is expected to regulate PI-3 kinase is a unique fashion. However, p55PIK binds to phosphorylated IRS-1, so it should be engaged during stimulation of responsive cells with insulin or IGF-1, IFN-α, IL-4, IL-9, growth hormone, or any other factor that stimulates tyrosine phosphorylation of IRS-1. Additional work should establish a unique role for p55PIK in growth and development.

ACKNOWLEDGMENTS

We thank Bruce Spiegelman for the F442a cDNA library, Jon Backer for an antibody against p110, Michael Waterfield for the p110 cDNA in a baculovirus, previous members of Ora Rosen's laboratory for the baculovirus containing IRβ, and Junichi Miyazaki for the CAGG expression vector.

This work was supported by grants DK 38712 and DK 43808 to M.F.W. and by a grant supporting the Diabetes and Endocrinology Training Center at the Joslin Diabetes Center (DK 36836). S.P. is a Fulbright Scholar, and T.A. and X.J.S. are fellows of the Juvenile Diabetes Foundation. M.G.M., Jr., was partially supported by the

Albert J. Ryan Foundation at Harvard Medical School and an NIH NRSA training grant (DK 07260) during the course of this work.

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